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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

First Quarterly Report of Progress

on

Research Project Number 4804-14-004

Order Number FDO-5013

July 1 - September 30, 1960

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## HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

### I. Introduction

Paralytic shellfish poison, which is produced by Gonyaulax catenella and may be isolated from toxic mussels or clams, is one of the most poisonous substances known to man. Its intermittent occurrence in shellfish growing areas along the Pacific and North Atlantic Coasts presents a difficult problem of detection and control to the public health agencies of the States involved (1). The fact that there is no effective medical treatment for paralytic shellfish poisoning makes it a particularly hazardous material to handle in the laboratory or elsewhere. Studies along biochemical and immunological lines have, therefore, been undertaken in the hope of contributing to the solution of these problems.

#### Purpose and Scope

The purpose of this project is (a) to determine the feasibility of joining the toxin of Gonyaulax catenella with other molecules to produce one or more conjugates having immunogenic properties, (b) to develop a specific micro-assay method for paralytic shellfish poison based on immunological reactions, and (c) to lay the ground work for immunization of humans against the poison.

It is clear from the objectives of this project that the research to be undertaken has two distinct, but interrelated, phases. One is concerned primarily with the chemistry of the poison and preparation of the antigen, while the other is concerned with the immunological

problems associated with the production of antibodies, demonstration of their presence, and evaluation of the haptenic properties of paralytic shellfish poison.

#### Properties of the Poison

Although paralytic shellfish poison has been isolated in an essentially pure form (2), its structural formula is unknown. Quantitative measurement of the poison currently is made either by a bio-assay procedure (3) or a chemical procedure (4). In neither case, however, are the methods entirely specific for the poison.

The purified natural poison is generally regarded as non-antigenic because of its relatively low molecular weight and the failure of experimental animals to become solidly immune after repeated exposure to non-fatal doses (5). Some unpublished evidence of increased resistance in rats has been obtained in this laboratory, but the biological mechanism has not been determined.

The empirical formula for paralytic shellfish poison is  $C_{10}H_{17}N_7O_4 \cdot 2HCl$  (6). Available evidence indicates that the molecule is heterocyclic and contains an imino nitrogen group ( $C = NH$ ) which is involved in tautomerism. The poison molecule resembles creatinine in many of its chemical reactions. It does not absorb light in the ultraviolet region; however, after oxidation under neutral or basic conditions, it shows two absorption maxima in this region(7).

## II. Experimental

On the basis of the foregoing information, preliminary investigations were begun to develop basic methods and to orient the research staff to

any technical difficulties which might be involved. For purposes of clarity the chemical and immunological studies are presented separately.

#### Chemical Studies Related to the Preparation of Antigens

Prior to undertaking any structural modifications of the poison molecule, preliminary studies were undertaken to learn something of the chemical reactivity of the nitrogen groups present. Conductometric titrations containing 1 milligram of the poison dihydrochloride were made with sodium hydroxide and methyl iodide. Although titrations of model compounds, such as pyridine and creatinine, with hydrochloric acid, followed by back titration with sodium hydroxide, were found to conform with the theory, the results obtained on the titration of the poison were uninterpretable. In view of the limited supply of poison, no further experimental work was undertaken at this time.

Studies were carried out on the diazotization and coupling of known aliphatic compounds using five to ten milligram quantities of reactants in order to gain proficiency in the use of micro and semi-micro techniques. Adaptations were made of the Curtius reaction which is used to prepare aliphatic diazo compounds (8). Sodium bromide was added, because it has been observed to catalyze diazotization and prevent self-coupling (9). Evidence of a reaction between the poison and nitrous acid was given by a change in the reaction mixture from colorless to bright yellow, with the retention of color even after the removal of excess nitrite ion by the addition of sulfamic acid and also by the subsequent coupling of the reaction

product with  $\beta$ -naphthol (traditional diazocoupling reagent) under basic conditions. The latter reaction appears to be very sensitive, with a discernible pink color being obtained in the presence of microgram quantities of the diazotized product. In view of the need for a method to follow the diazotization and coupling reactions of paralytic shellfish poison, studies are underway to quantitate this color reaction.

Although the following experimental work has not been confirmed, the implications of the observation make it worthwhile to relate briefly. When diazotized paralytic shellfish poison was reacted with 2-4 dinitrophenylhydrazine reagent, an orange precipitate was formed (not excess reagent) which was presumed to be a hydrazine of the diazotized poison. If this be the case, it would appear that the presence of the diazo-group on the poison molecule gives it an internal stability not normally present since paralytic shellfish poison does not normally react with 2-4 dinitrophenylhydrazine.

In order to get the immunological studies underway, an attempt was made to prepare a paralytic shellfish poison-protein "antigen." After using the above procedure for preparing the diazotized poison, and removing the excess nitrite ion by the addition of sulfamic acid, it was then given an opportunity to couple with ovalbumin by mixing at pH-7. Although no methods were available to judge the degree of coupling, this material was used as the first antigen preparation in immunological studies discussed below.

#### Immunological Studies Related to Determining the Antigenicity of Shellfish Poison Complexes

Prior to working with the first poison conjugate provided by

the chemistry laboratory, tests were made with a known haptenic system. The antigen consisted of diazotized histamine coupled to horse serum, which is available commercially under the trade name, "Hapamine."

Two groups of two rabbits each were immunized. One group received intraperitoneal injections of full strength antigen, and the second group received intravenous injections (lateral ear vein) of a 1:10 dilution of antigen in saline. Initial injections consisted of 0.1 ml doses followed by increased volumes given at two-dose intervals over a 10-day period. The final injection consisted of 1 ml. The animals were bled by cardiac puncture 7 days following the final injection. The sera were collected and titrated for antibody content.

The sera were tested for antihapamine precipitins by mixing a constant volume of each serum with varying concentrations of antigen and observing for precipitate. The titers of the sera were found to be in excess of 1:4000 and "equivalence zones" occurred in the 1:200 to 1:300 dilutions.

The haptenic property of histamine was demonstrated by employing a "haptene inhibition test" in which excess histamine alone is mixed with antihapamine serum and allowed to react. Histamine saturates the antihapamine molecule during this initial reaction in such a way that the antihapamine serum is not capable of giving a precipitin reaction when Hapamine is later added to the system. The inhibition by histamine of the reaction between the Hapamine-antihapamine system, described above, gave evidence for the haptenic nature of histamine.

After completion of the Hapamine studies, a similar experiment was undertaken using the shellfish toxin-protein antigen described in

the previous section. This preparation contained 2.75 milligrams of ovalbumin per milliliter and diazotized paralytic shellfish poison equivalent to 0.322 milligrams per milliliter. Although the toxicity of diazotized poison was found by mouse test to be less than one percent of the original poison, the antigen solution was sufficiently toxic to cause problems on injection into rabbits. Both of the rabbits in the intravenous series and one in the intraperitoneal series were killed during course of injections. For the lone survivor, it was possible to administer only a total of 2.5 milliliters of the antigen preparation.

One week after final injection the surviving rabbit was bled and the serum collected. Using doubling dilutions of antigen from 1:2 to 1:1024 against a 1:5 dilution of serum, no precipitin reaction was observed. Similar titrations were attempted using unmodified ovalbumin and paralytic shellfish poison singly, and likewise no precipitin reactions were noted.

The lack of a demonstrable antibody titer may be accounted for in part by the following mechanisms: (a) the total protein antigen injected was not sufficient to elicit high antibody titers; (b) if free toxin were present in significant amounts in the material injected, an in vivo binding of antibody might occur; (c) in vitro binding of antibody to free toxin might have occurred, thus preventing a precipitin reaction between toxin-protein antigen and anti-toxin protein (haptene-inhibition). In view of the lack of reaction when ovalbumin was titrated against the antiserum, (a) above appears to be most probable explanation.

### III. Projected Research for Second Quarter, FY 1961

The two problems of immediate concern in connection with the chemical aspects of this project are (a) the development of a quantitative procedure for measuring the extent of diazotization of paralytic shellfish poison and the degree of coupling of the reaction product with proteins or other agents and (b) the development of techniques for decreasing the toxicity of paralytic shellfish poison-protein preparation without serious alteration of its antigenic properties.

In connection with the immunological work, additional studies will be undertaken on the immunogenic properties of the paralytic shellfish poison antigen, along with appropriate control studies to evaluate the antigenic properties of the native protein, altered proteins, and perhaps the diazotized paralytic shellfish poison alone.

### IV. Summary

In addition to completing orientation studies with non-toxic simulants for paralytic shellfish poison and a known haptenic system based on "Hapamine," preliminary evidence was obtained which suggests the formation of a stable diazo-derivative of the poison. This compound was given an opportunity to combine with ovalbumin, and the complex was used as the first experimental antigen. Failure of this preparation to elicit antibodies in rabbits was probably due to the fact that its toxicity prevented injection of sufficient antigen to develop a detectable titer. Plans are outlined for further chemical and immunological studies designed to overcome this difficulty.

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